Comparisons of Five *Saccharomyces cerevisiae* Strains for Ethanol Production from SPORL-Pretreated Lodgepole Pine

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The performances of five yeast strains under three levels of toxicity were evaluated using hydrolysates from lodgepole pine pretreated by Sulfite Pretreatment to Overcome the Recalcitrance of Lignocelluloses (SPORL). The highest level of toxicity was represented by the whole pretreated biomass slurry, while intermediate toxicity was represented by the hydrolysate with partial loading of pretreatment spent liquor. The zero toxicity was represented using the enzymatic hydrolysate produced from thoroughly washed SPORL lodgepole pine solids. The results indicate that strains D5A and YRH400 can tolerate the whole pretreated biomass slurry to produce 90.1 and 73.5% theoretical ethanol yield. Strains Y1528, YRH403, and FPL450 did not grow in whole hydrolysate cultures and were observed to have lower ethanol productivities than D5A and YRH400 on the hydrolysate with intermediate toxicity. Both YRH400 and YRH403 were genetically engineered for xylose fermentation but were not able to consume xylose efficiently in hydrolysate. © 2014 American Institute of Chemical Engineers *Biotechnol. Prog.*, 30:1076–1083, 2014
Keywords: yeast fermentation, inhibitors, toxicity, pretreatment, ethanol

Introduction

Cellulosic ethanol has several advantages in terms of energy security, reduction in greenhouse gas emissions, and economic (especially rural) development,[1,2] despite embodying greater technical risks and capital outlays than traditional grain ethanol processes. Woody biomass is an important renewable resource potentially available for conversion to biofuels. Forest resources, including managed forest and woody waste, could supply approximately 300 million dry tons per year.[3] Woody biomass is attractive because a logistical network already exists for its collection and transportation. Woody biomass also allows for flexible harvest, which reduces the need for long term storage. Its high density relative to herbaceous biomass reduces transportation costs. Low ash content eases maintenance of process equipment.[4] Also, one can envision, that pulp mills could be expanded for ethanol fermentation, thereby, lowering technical risks and possibly capital requirements.

One drawback to processing woody biomass is that it is more resistant to processing than herbaceous biomass. As a result, few pretreatment strategies have been shown to give satisfactory yields from woody biomass.[4] Traditional pretreated strategies applied to wood include alkaline, sulfuric acid catalyzed steam explosion, and organosolv. A more recently developed method is sulfite pretreatment to overcome recalcitrance of lignocelluloses (SPORL).[5] This pretreatment reported substantially higher sugar yields than the previously cited methods and would be much easier to scale because all the unit operations are based upon those used in sulfite pulping. Reliance on traditional unit operations is a very effective strategy for reducing technical risk.

As an aqueous pretreatment, SPORL produces liquid and solid process fractions. The solids contain largely cellulose, which is subsequently digested to glucose by applying enzymes (e.g., cellulases and β-glucosidase). The liquor

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Additional Supporting Information may be found in the online version of this article.

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Table 1. Chemical Compositions of the Double-Washed Solids and Concentrated Spent Liquor

Washed Solids (%)		Concentrated Spent Liquor (g/L)	
Klason lignin	37.3 ± 0.9	Arabinose	5.8 ± 0.4
Arabinan	ND	Galactose	13.0 ± 0.3
Galactan	ND	Glucose	36.2 ± 1.6
Glucan	53.3 ± 0.5	Xylose	18.0 ± 0.6
Xylan	1.1 ± 0.2	Mannose	47.0 ± 1.3
Mannan	0.9 ± 0.1	Furfural	0.5 ± 0.0
		HMF	6.4 ± 0.5
		Acetic acid	6.6 ± 0.2

ND: not detectable.

stream contains primary water and mono sugars, Lignosulfonate and other soluble products. Process simplification demands mixing the two streams prior to enzymatic saccharification and fermentation of all the sugars. Furthermore, lignosulfonate, especially those from SPORL process, can enhance enzymatic saccharification of pretreated solids by preventing nonproductive binding of cellulase to lignin on solid substrate.[6–8] Fermentation of spent sulfite liquor from softwoods that contains a significant amount of lignosulfonate and phenolic compounds with relatively low amounts of furans and acetic acid has long been in industry practice, which suggests that strains of *Saccharomyces cerevisiae* can tolerate phenolic compounds. Fermentation of spent sulfite liquor from hardwood species using xylose fermenting strains, however, are very challenging due to the presence of significant amounts of acetic acid, lignosulfonate and phenolic compounds, coupled with low xylose concentration.[9,10]

We have recently published several studies optimizing SPORL pretreatment and enzymatic digestion using lodgepole pine as the feedstock.[11–14] In the first, the cellulose and liquor streams were fermented separately to produce 276 L of ethanol per metric ton of wood or 72% of the theoretical limit.[13] Potential inhibitory chemicals were removed from the liquid stream by passing it through a packed bed containing the adsorbent XAD4. While the resin can be regenerated,[15] its use and the separate fermentations add expense. To streamline the process, simultaneous enzymatic saccharification and combined fermentation of the enzymatic hydrolysate with the liquor was carried out but at a low solids of 10% using specially adapted *S. cerevisiae* yeast strain Y5.[11] Conditioning the liquor prior to fermentation by over-liming or XAD absorption improved ethanol productivity, but it did not alter the final ethanol yield. This combined fermentation approached was later extended to high solids fermentation of SPORL pretreated softwood hydrolysate using YRH400.[12,14] Recently, we also determined that the dissolved lignosulfonate has potential as a marketable coproduct as a dispersant.[14]

In this study, five *S. cerevisiae* yeast strains including two xylose fermenting strains[16] were evaluated using SPORL pretreated lodgepole pine at various inhibitor concentrations. Rather than focusing on producing high titer ethanol as we demonstrated previously,[14] we used a lower inoculum volume–the initial cell optical density[17] $OD_{600\ nm}$ of 3.5 to conduct fermentation at approximately 15% water insoluble solids. Less yeast inoculum is a more stringent test of yeast tolerance to inhibitors. *Saccharomyces* strains are variable in respect to fermenting galactose and incapable of fermenting xylose or arabinose. The molecular engineering of *S. cerevisiae* that ferment either xylose or xylose and arabinose has been a source of immense activity for the past two decades.[18] The two xylose fermenting strains were chosen in part because both were constructed using commercial yeasts. In particular, one of the parental yeasts (Y-1528) has previously been recommended for fermentation of softwood hydrolysates because it has the rare ability, among yeasts, of cofermenting glucose and galactose.[19] As controls for xylose fermentation, the two parental strains and a third native industrial *S. cerevisiae* strain were evaluated as well.

Materials and Methods

Materials

Lodgepole pine wood chips were produced at the Forest Products Laboratory (Madison, WI) from a mountain pine beetle-killed tree harvested from the Canyon Lakes Ranger District of the Arapaho-Roosevelt National Forest (CO). Detailed information about the tree, harvesting, and transportation have been described previously.[20] The wood chips were screened to retain chips between 6 and 38 mm. The screened chips were frozen at approximately −16°C until use.

Commercial cellulase enzymes Cellic® CTec2 (abbreviated CTec2) was generously provided by Novozymes North America (Franklinton, NC). The cellulase activity was 147 FPU/mL. Sodium acetate buffer, sulfuric acid, and sodium bisulfite were used as received from Sigma-Aldrich (St. Louis, MO). All chemicals were ACS reagent grade.

SPORL substrate production

The accepted lodgepole pine wood chips of 2 kg in oven dry weight (od) were pretreated using a dilute sulfite solution at liquor to wood ratio 3:1 at 180°C for 20 min. The sodium bisulfite and sulfuric acid loadings were 8 and 2.2 wt% on an od wood basis, respectively. Pretreated wood chips and the spent liquor were combined and disk milled together with the addition of water (2.26 L/kg untreated wood) to facilitate milling and as prewashing of the solids. The resultant slurry was pressed to separate into a solid and a liquor fraction. The solid fraction was then washed again thoroughly using deionized (DI) water. The resultant solid substrate was used as an inhibitor-free control for fermentation experiments. The liquor fraction was concentrated through vacuum evaporation at 52°C for high solids processing. Losses of fermentable sugars were minimal and the reduction of major inhibitor 5-hydroxylmethyfurfural (HMF) was only approximately 15% from the concentration step.[12] The reductions in furfural and acetic acid was approximately 60%, however, both are minor fermentation inhibitors as the spent liquor was produced using softwood lodgepole pine. Therefore, changes in their concentrations should not affect the integrity of the study. Detailed descriptions of the substrate production process can be found in a previous study.[12] The chemical compositions of the double-washed solids and the concentrated liquor were analyzed and listed in Table 1.

Microorganism and culture

Descriptions of the five strains used in the present study are shown in Table 2. The strains were grown at 30°C for 2 days on YPD agar plates containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L agar. A colony from the plate was transferred by loop to liquid YPD

Table 2. List of Yeast Strains

Strain	Genotype	Reference
D5A	*S. cerevisiae* isolated from cheese whey	ATCC
YRH400	D5A(*KanMX4*; P_{PGK1}-*XYL1*-T_{PGK1}; P_{ADH1}-*XYL2*-T_{ADH1}; P_{HXT7}-*XKS1*-T_{HXT7})	Hector et al.[16]
Y1528	*S. cerevisiae*, natural galactose-assimilating isolate	USDA-ARS
YRH403	Y-1528 hoΔ::(*KanMX4*; P_{PGK1}-*XYL1*-T_{PGK1}; P_{ADH1}-*XYL2*-T_{ADH1}; P_{HXT7}-*XKS1*-T_{HXT7})	Hector et al.[16]
FPL450	*Saccharomyces carlsbergensis*	ATCC9080

medium in a flask and cultured overnight at 30°C with agitation at 90 rpm on a shaking bed incubator (Thermo Fisher Scientific, Model 4450, Waltham, MA). The biomass concentration was monitored by optical density at 600 nm (OD_{600}) using a UV–Vis spectrometer (Model 8453, Agilent Technologies, Palo Alto, CA). The time-dependent optical density profiles of the five strains were shown in Supporting Information Figure S1. The cultured medium at 72 h (with an average optical density at 600 nm of approximately 30) was used to inoculate the fermentation culture.

Analytical methods

The chemical compositions of the pretreated lignocellulose and the concentrated spent liquor were analyzed as described previously.[20] The lignocellulose sample was ground in a Wiley mill (Model No. 2; Arthur Thomas Co, Philadelphia, PA) to 20 mesh (~1 mm) and hydrolyzed in two stages using sulfuric acid of 72% (v/v) at 30°C for 1 h and 3.6% (v/v) at 120°C for 1 h. Carbohydrates of the hydrolysates were analyzed by high performance anion exchange chromatography with pulsed amperometric detection (ICS-5000, Dionex). Klason lignin (i.e., acid insoluble lignin) was quantified gravimetrically.

Monosaccharides (glucose, mannose, xylose, arabinose, and galactose) were determined using a Dionex HPLC system (Ultimate 3000) equipped with an RI (RI-101) and UV (VWD-3400RS) detector and BioRad Aminex HPX-87P column (300 mm × 7.8 mm) operated at 80°C. Double distilled water was used as eluent at a flow rate of 0.6 mL/min. Inhibitors (acetic acid, formic acid, furfural, and HMF) and ethanol were measured by the same HPLC system equipped with BioRad Aminex HPX-87H column (300 mm × 7.8 mm) operated at 60°C. Diluted sulfuric acid solution of 5 mM was used as eluent at a flow rate of 0.6 mL/min. All sample injection volume was 20 μL. Samples were diluted in distilled water, and filtered by a 0.22 μm filter prior to injection.

Quasi-simultaneous enzymatic saccharification and combined fermentation

Different volumes of the concentrated liquor and make-up water were mixed with a fixed amount of double-washed pretreated solids to create three hydrolysates with a 15% solids (water insoluble) loading. The formulation of these three mixtures (I, II, and III) are listed in Table 3. The loadings of concentrated liquor in the three mixtures were 0 (III), 0.94 (II), and 1.65 (I) g/g double-washed solids (Table 3). The liquor loading of 1.65 is the same ratio for the pretreated biomass prior to separation of solids and liquid. Therefore, Sample I represents the whole pretreated biomass slurry when diluted to 15% solids. The liquefied samples by cellulase represented combined pretreatment and enzymatic hydrolysates that have different levels of fermentation inhibitors (Table 3) and were used to study yeast strain performance for ethanol production through fermentation. Duplicate fermentation runs were carried out. The standard deviations were used as error bars in plotting.

Table 3. Make-Ups of and Composition of Pretreated Sample Mixtures Used for Enzymatic Saccharification and Fermentation

Sample No	I	II	III
Formulation			
Washed Solids in od weight (g)	4.90	4.90	4.90
Concentrated Liquor (g)	8.09	4.61	0
Liquor loading (g/g od washed solids)	1.65	0.94	0
Water insoluble solids (%)	15	15	15
Composition (g)*			
Arabinan	0.07 (1.40)	0.04 (1.07)	0.00 (ND)
Galactan	0.17 (3.11)	0.09 (1.85)	0.00 (ND)
Glucan	2.75 (38.4)	2.70 (37.0)	2.61 (34.4)
Mannan	0.70 (12.4)	0.40 (8.04)	0.04 (ND)
Xylan	0.30 (5.58)	0.18 (3.47)	0.06 (0.81)
Furfural	0.00 (ND)	0.00 (ND)	0.00 (ND)
HMF	0.02 (1.21)	0.01 (0.42)	0.00 (ND)
Acetic acid	0.02 (4.90)	0.01 (2.30)	0.00 (1.83)
Formic acid	(0.28)	(ND)	(ND)

*Numbers in the parenthesis are the either the inhibitors or the corresponding component monomeric sugar concentrations in g/L based on measurements in combined hydrolysates derived from saccharification of the mixtures prior to fermentation; ND: not detectable.

Quasi-simultaneous enzymatic saccharification and combined fermentation (SSCombF) of the enzymatic hydrolysate of the pretreated lodgepole pine solids and spent liquor were carried out in 250 mL Erlenmeyer flasks using a shaker/incubator (Thermo Fisher Scientific, Model 4450, Waltham, MA). Each mixture was adjusted to pH 6.2 using solid calcium hydroxide and the pH was controlled by adding acetic acid/sodium acetate buffer (50 mM) of pH 5.5. An elevated pH 5.5,[21,22] higher than commonly used pH 4.8–5.0, and the application of lignosulfonate in SPORL pretreatment liquor can significantly reduce nonproductive cellulase binding to lignin to enhance lignocellulose saccharification.[6–8] The enzymatic hydrolysis was conducted at 15% solids (water insoluble) using CTec2 at 15 FPU (or 0.1 mL)/g glucan. Liquefaction of solid substrate was observed within approximately 16 h at 50°C and 200 rpm. The compositions of the liquefied samples were analyzed. The samples were then cooled down to 35°C and the shaker speed was reduced to 90 rpm and inoculated with yeast seed. The initial optical density of the yeast for all fermentation experiments was controlled at $OD_{600} = 3.5$. No additional nutrients were applied during fermentation. Samples of the fermentation broth were taken periodically for monosaccharides, inhibitors, and ethanol analyses.

Result and Discussions

Fermentation of SPORL pretreated combined hydrolysates by five yeast strains

The hydrolysates mixtures I, II, III contained primarily glucose but also significant amounts of mannose, galactose,

Table 4. Glucose Consumption, Ethanol Productivity, and HMF Metabolization in the First 24 h (Unless Indicated) During Fermentation of the Three Combined Hydrolysates Using Five Yeast Strains

	I (g/L/h)			II (g/L/h)			III (in first 8 h; g/L/h)	
Yeast	Glucose	Ethanol	HMF	Glucose	Ethanol	HMF	Glucose	Ethanol
D5A	−1.525	0.759	−0.046	−1.570	0.943	−0.019	−2.284	1.516
YRH400	−0.733	0.309	−0.023	−1.567	0.838	−0.017	−2.506	1.611
Y1528	0.027*	0.061	−0.005	−0.049	0.170	0.003†	−1.481	1.286
YRH403	0.038*	0.063	−0.005	−0.179	0.198	−0.003	−1.941	1.222
FPL450	−0.013	0.076	−0.005	−0.786	0.694	−0.015	−2.044	1.222

*No glucose consumption. The small positive numbers were resulted from fittings of the experimentally measured glucose concentrations that contain uncertainties.

†No HMF metabolization. The small positive number was resulted from fittings of the experimentally measured glucose concentrations that contain uncertainties.

xylose, and arabinose, especially for I and II, as shown in Table 3. The hydrolysates also contained side-products that are known to have antimicrobial activity, including acetic acid and HMF. Furfural was effectively removed by the liquor concentration process, which used vacuum evaporation.

Fermentation performances of the five yeast strains (Table 2) were compared using the three SPORL hydrolysate mixtures. Two (YRH400 and YRH403) were *S. cerevisiae* strains engineered for xylose fermentation by expressing xylose reductase and xylitol dehydrogenase from *Scheffersomyces stipitis* and over expressing the native xylulose kinase.[16] These *S. cerevisiae* strains had been earlier reported to produce approximately 15% more ethanol compared to the parental strains from ammonia pretreated switchgrass because of their ability to ferment xylose.[16] The other three strains are nonengineered and therefore unable to ferment xylose. Two of these strains (D5A and Y1528) are the parents for the engineered strains and the last (FPL450) has been used previously in this laboratory.[23]

Fermentation results from using each of the five yeast strains are summarized in Table 4. Only *S. cerevisiae* strain YRH400 and its parental strain D5A were observed to grow in the most challenging hydrolysate I (the pretreated whole biomass slurry). These two strains used almost all of the glucose and mannose and produced 22.96 and 28.14 g/L of ethanol, respectively (Figures 1a and 2a). Disappointingly, YRH400 did not ferment the xylose (Figure 2a), despite being specifically engineered for its metabolism. For this reason, in part, the native parental strain D5A outperformed YRH400. The remaining three yeasts failed to ferment any of the sugars including glucose and produced only trace amounts of ethanol between 1.52 and 1.82 g/L (Figure 1a). The reason YRH400 did not use xylose and three of the other strains (Y1528, FPL450, and YRH403) used no sugars was because of inhibitors present in the hydrolysate I and the low concentration of xylose in the hydrolysates.

When the concentration of inhibitors presented to the yeasts was decreased, by using hydrolysate II instead of I, all five strains fermented glucose and mannose to produce ethanol (Figures 1b and 2b). However, the sugar consumption rates of D5A and YRH 400 were much faster than FPL450, YRH403 and Y1528 (Table 4). The ethanol concentration at 96 h were 26.4, 25.2, 25.6, 20.8, and 15.1 g/L for D5A, YRH400, FPL450, YRH403, and Y1528 (Figure 1b), respectively. It appears that when using strains YRH403 and Y1528, prolonged fermentation times can increase ethanol production.

When inhibitor concentrations were reduced to zero by using enzymatic hydrolysate (III), made from double-washed solids, the sugar consumption rates of D5A and YRH400 appeared slightly faster than the other strains (Table 4). The final ethanol yields of the five strains were all similar (Figure 1c).

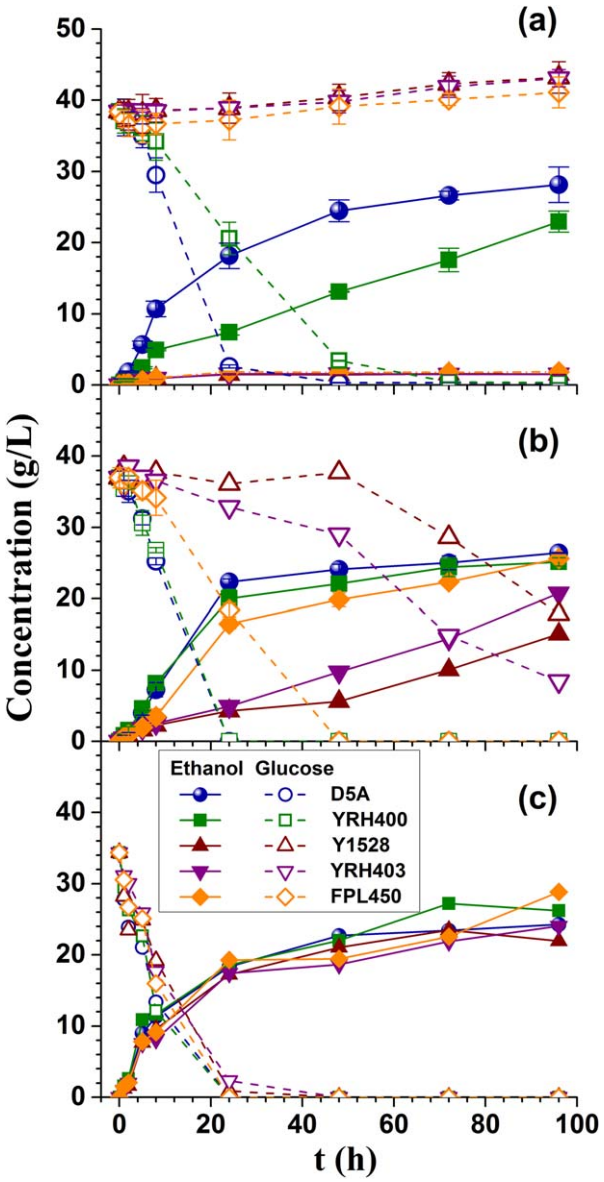

Figure 1. Time-dependent ethanol and glucose concentrations during fermentation of the three combined hydrolysates using the five yeast strains. (a) Hydrolysate I; (b) hydrolysate II; and (c) hydrolystae III.

Fermentation inhibitors

Organic inhibitors are grouped based upon their functional groups as aldehydes, aromatics, and weak organic acids.[24] Aldehydes include furfural and HMF, which are generated from degradation of pentose and hexose sugars, respectively.

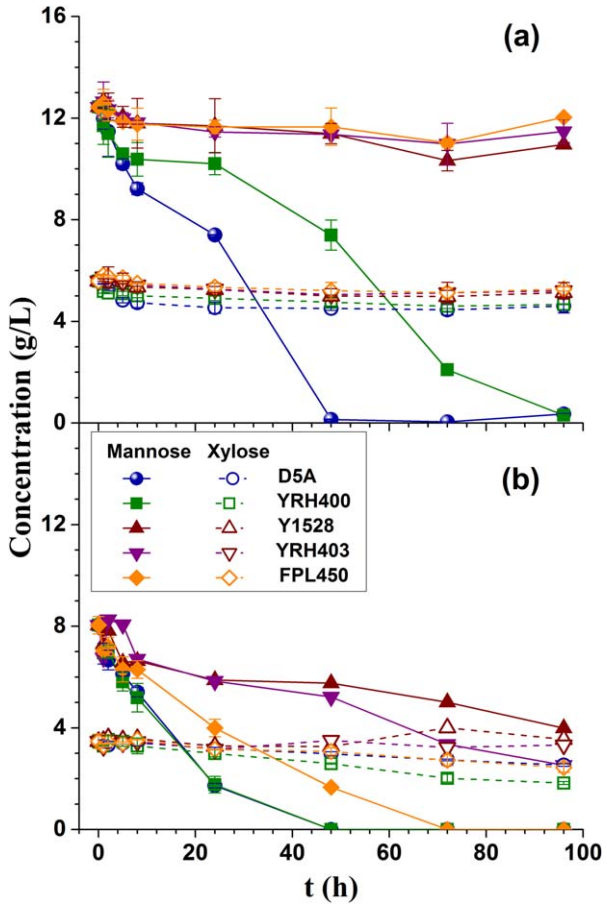

Figure 2. Time-dependent mannose and xylose consumption during fermentation of two combined hydrolysates using the five yeast strains. (a) Hydrolysate I and (b) hydrolysate II.

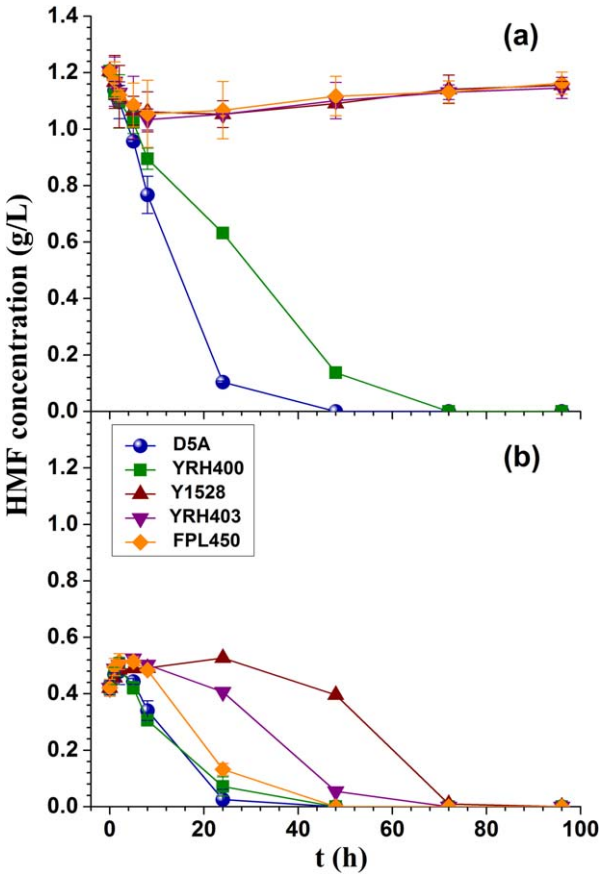

Figure 3. Time-dependent HMF metabolization during fermentation of two combined hydrolysates using the five yeast strains. (a) Hydrolysate I and (b) hydrolysate II.

Organic acids include acetic, formic, and levulinic. Formic and levulinic acids are formed in equal molar ratios when HMF further decomposes. Acetic acid is released from hemicellulose and aromatics from lignin during pretreatment. In this study, acetic acid, formic acid, and HMF were monitored (Table 3). Lodgepole pine as softwood used in the present study has a low xylan content, which resulted in a very low amount of furfural formation during pretreatment. The vacuum evaporation of the pretreatment hydrolysate (spent liquor) for concentration further diminished furfural to an undetectable concentration.[12]

Furans inhibit enzymes involved in metabolism and generate reactive oxygen species.[25] Microbial cells respond to furfural and HMF by reducing them to their corresponding less toxic alcohol forms furfuryl and HMF alcohols.[26,27] Furans can lead to prolonged lag phases and lower ethanol productivities and yields. In *S. cerevisiae*, alcohol dehydrogenases (ADH) 6 and 7 appear to be most active toward furans and over expression has been shown to increase furan tolerance in yeast.[28] Using a higher inoculum also enhances furan tolerance, presumably by increasing the gross rate of furan reduction.[29]

There is direct evidence that the presence of inhibitors caused no growth in three of the yeast cultures. The rate of HMF consumption for all five yeast cultures is calculated in Table 4. The successful yeast cultures steadily and completely consumed HMF for hydrolysate I (Figure 3a). In contrast, HMF concentration remained steady for the stalled fermentations using hydrolysate I (Figure 3a). This does not imply that HMF was solely responsible for stalling the fermentations, but rather the combined effect of all the inhibitors overwhelmed the cells. For hydrolysate II, because the concentrations of all inhibitors were lower than I, all five strains could tolerate the inhibitors and produce ethanol, which is also reflected by the complete consumption of HMF (Figure 3b).

Results for the lack of growth with Y1528 using hydrolysate I appear at odds with earlier observations for this strain.[30] In the prior study, the strain was shown to grow in rich medium supplemented with glucose spiked with either furfural (1.6 g/L), HMF (4 g/L), or acetic acid (15 g/L at pH 6.0) and inoculated at 4 g DCW/L. In the present study, the hydrolysate included 4.9 g/L acetic acid (at pH 5.6), 0.28 g/L formic acid, 1.2 g/L HMF, ~2 g DCW/L, and no additional nutrients. That Y1528 failed to grow in our cultures relates to the complexity of the hydrolysate, which included unmeasured inhibitors such as aromatics and uronic acids.[31] When the amount of inhibitors decreased, Y1528 could grow, albeit slowly, as shown in Figure 1b. Also notable, another study that included a related yeast strain observed that the growth rate fell by 50% at 4 g/L acetic acid and ceased at approximately 8 g/L.[25] Results from this later study regarding sensitivity to acetic acid concur with results presented here. The mechanism for acetic acid inhibition of yeast is well described in the literature. In their undissociated form, the acids transverse the cellular membrane and disassociate, lowering the pH of the cytoplasm. Cells respond by

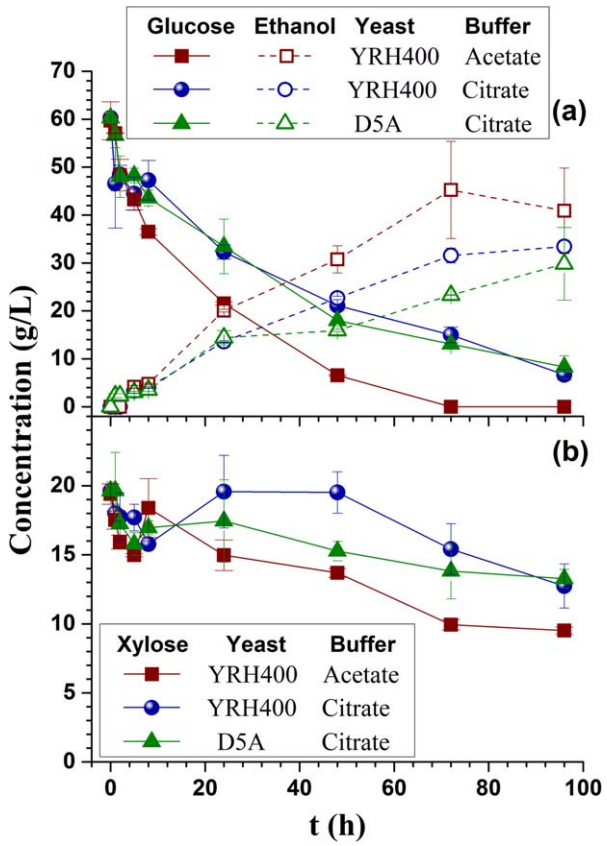

Figure 4. Comparisons of fermentation performance between YRH400 and D5A using a pure sugar solution at two different buffers. (a) Time-dependent glucose consumptions and ethanol productions and (b) time-dependent xylose consumptions.

exporting out the excess protons.[32–34] At low concentrations (up to 100 mM), organic acids can favor ethanol yield over biomass as cells generate excess ATP to maintain their pH, albeit at lower productivities. There is a linear relationship between undissociated acetic acid concentration and falling growth rates.[25] As the concentration of the undissociated acid increases in the culture, the cell becomes overwhelmed leading to acidification of the cytoplasm and eventually cell death. Intracellular accumulation of the anion may add further stress to the cell.[35]

Fermentation profiles for D5A and YRH400

While both yeast fermented the glucose in hydrolysate I, D5A fermented it faster (Table 4). Strain D5A consumed all of the glucose within 30 hr and YRH400 within 50 hr, as shown in Figure 1a. Therefore, it would appear that constitutive expression of the genes for xylose fermentation might interfere with glucose metabolism for this hydrolysate. On rich medium with pure glucose solutions (2%, w/v), it was reported D5A and YRH400 had similar ethanol productivities.[16] YRH400 was also previously reported to increase ethanol concentration by 14% compared to D5A when ammonia-pretreated switchgrass hydrolysate was used.[16] In that study, inhibitor concentrations were low and significantly more xylose was present.

Furthermore, when cultured on glucose, the xylose-fermenting strain 259ST grew approximately 10% slower than its parent strain 259A.[25] In yet another study, expression of several genes for xylose metabolism was demonstrated to decrease the specific growth rate on glucose medium by 13–47%.[36] The results from these previous studies, coupled with our results, suggest that over expression of certain genes for xylose metabolism may decrease ethanol productivity and yield from glucose. Perhaps expression of the xylose metabolism genes reduces the overall energy state of the cell; for example, xylulose kinase consumes ATP and it is known that over expression is detrimental to growth of yeast cells.[37]

Both strains fermented mannose more slowly than glucose. Mannose was used-up in 48 h and 96 h (Figure 2a) for D5A and YRH400, respectively. Mannose fermentation speeded up once glucose was near exhaustion (Figure 2a). Mannose utilization is not repressed by glucose but both sugars use the same transporters, which show a greater affinity for glucose.[38]

Glucose consumption and ethanol production rates for YRH400 and D5A were similar when inhibitors were reduced, as observed in hydrolysate II fermentations (Figure 1b). Mannose was consumed within 48 h for both D5A and YRH400, and 72 h for FPL450 (Figure 2b).

Xylose was not fermented by either strain using hydrolysate I (Figure 2a). While this result was expected for D5A, it was not for YRH400, which is engineered to ferment xylose. As a further control, D5A and YRH400 were challenged with a pure sugar mixture of glucose (60 g/L), xylose (20 g/L), galactose (10 g/L), arabinose (5 /gL), and mannose (20 g/L). In this case, YRH400 did produce 12% more ethanol than D5A, though xylose consumption was slow and incomplete (Figures 4a,b). The presence of other inhibitors in the hydrolysate and ethanol are likely also to impede xylose metabolism.

The failure of YRH400 cultures to ferment xylose in the hydrolysate cultures can be attributed to the presence of organic acids, furans, and the high ratio of glucose to xylose concentrations. Xylose fermentation is particularly susceptible to organic acid inhibition.[25,28] The acetic acid concentration for the hydrolysate I YRH400 culture can be calculated using the Henderson-Hasselbalch equation (using pH = 5.0, acetate = 4.9 g/L) as approximately 1.7 g/L. When adding in the effect of formic acid (at 0.28 g/L), the total organic acid concentration equals to levels previously observed to inhibit xylose fermentation.[32] However, it is not just acetic acid. When YRH400 was grown on the same sugar mixture described earlier using either acetate or citrate buffer; the acetate buffered culture outperformed that containing citrate (Figure 4a). Other inhibitors to xylose-fermentation that were present in the hydrolysates included furfural and HMF.[28] Additionally, xylose uptake into the cell occurs through hexose transporters and is inefficient at low xylose concentrations, especially in the presence of glucose. Low xylan levels present in softwood resulted in xylose concentrations in the hydrolysates (5–37 mM) that are significantly lower than the estimated K_M for xylose uptake in *Saccharomyces* yeasts (80–200 mM).[39–41] Since xylose uptake occurs through glucose transporters, when glucose is also present at high concentration, glucose is preferentially transported and xylose is not fermented.[42] The combined effect of all of these can be observed for experiment II, where even though the amounts of inhibitors were greatly reduced compared to experiment I, xylose was still not fermented by the YRH400 culture (Figure 2b).

When inhibitor level was high (hydrolysate I), galactose was not fermented by D5A or YRH400 (Figure 5a) even

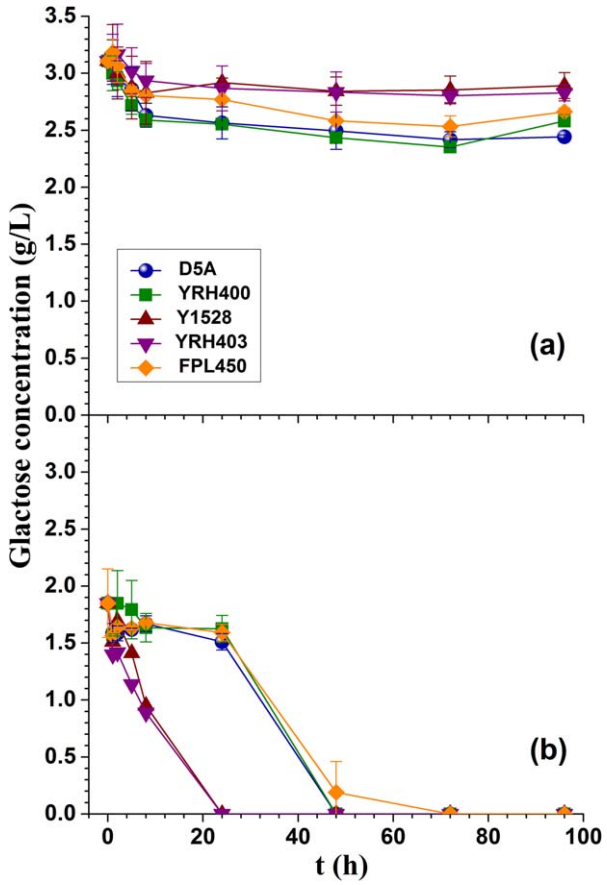

Figure 5. Time-dependent galactose consumption during fermentation of two combined hydrolysates. (a) Hydrolysate I and (b) hydrolysate II.

though D5A ferments galactose.[43] Galactose is metabolized via the Leloir pathway,[38] which is repressed in the presence of glucose. Galactose metabolism is observed to be induced following a long lag phase after glucose has been exhausted from the culture. The interaction of glucose repression for much of the fermentations followed by the continued presence of inhibitors (weak organic acids and ethanol) probably prevented induction of the Leloir pathway. In an earlier study, when D5A was grown on a pure glucose and galactose mixture (7.5%, w/v of each), it failed to consume the galactose after exhausting the glucose.[43] This article also reported on construction of noncatabolite mutants able to coferment glucose and galactose, which might be a future solution. Alternately, *Saccharomyces* Y1528 was included because it was reported to have the rare ability to coferment glucose and galactose in both mixed sugars and hydrolysate type cultures.[19,44] Unfortunately, neither Y1528 nor YRH403 tolerated the SPORL hydrolysate I (Figure 5a). At the lower inhibitor levels in hydrolysate II, all five yeast strains consumed more than a minor amount of the available galactose after exhausting the glucose (Figure 5b). In addition, the results indicate that galactose consumption rates for Y1528 and YRH403 are faster than D5A and YRH400. As expected, D5A and YRH400 only began to consume the galactose after exhausting the glucose.[43] In summary, glucose and galactose were successfully fermented using strains D5A and YRH400. Fermentation of xylose remains problematic. However, current research of incorporating a more efficient xylose metabolic pathway and higher affinity transporters hold promise for future strain improvements.[39,45–47]

Concluding Remarks

Fermentation of the SPORL whole hydrolysate was successful without conditioning when using strains D5A and YRH400. The ethanol yield efficiencies based upon glucan content were 90.1 and 73.5% of theoretical. Unfortunately, YRH400, engineered to metabolize xylose, did not ferment xylose when challenged with SPORL generated hydrolysates, though it did in simulated sugar mixtures. Xylose fermentation by recombinant *S. cerevisiae* strains has been reported to be particularly sensitive to the presence of inhibitors associated with hydrolysates. Still the overall ethanol yield compares favorably with those reported previously for softwoods. Incorporating a more efficient xylose metabolic pathway and higher affinity transporters are approaches for developing future xylose fermentation strains.

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